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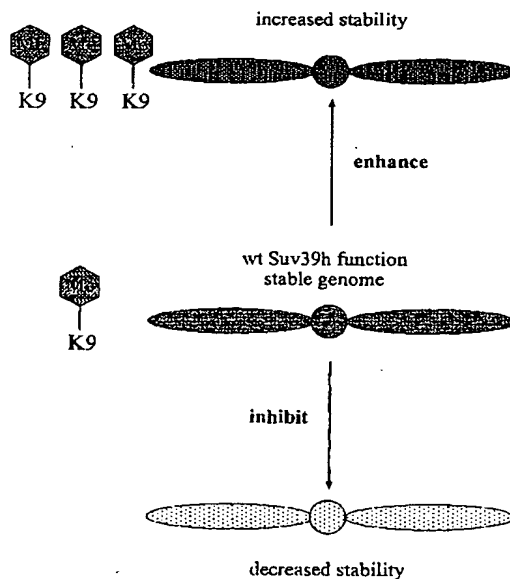
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(54) Method for identifying compounds modifying methyltransferase-dependent chromosome stability

(57) A method for identifying compounds that alter higher order chromatin dependent chromosome stability is based on determining the compounds' ability to

modify a methyltransferase with Suv39h-like methyltransferase activity. The identified compounds are useful in therapy, in particular the therapy of human cancer.

Fig. 8



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Description

[0001] The invention relates to a method for identifying compounds influencing chromosome dynamics in eukaryotic cells. In particular, the invention relates to the treatment and prevention of human conditions by modulating higher order chromatin dependent chromosome stability.

[0002] Higher-order chromatin is essential for epigenetic gene control and for the functional organisation of chromosomes. Differences in higher-order chromatin structure have been linked with distinct covalent modifications of histone tails which regulate transcriptional 'on' or 'off' states (Grunstein, 1998; Turner, 1998; Strahl and Allis, 2000) and influence chromosome condensation and segregation (Karpen and Allshire, 1997; Wei *et al.*, 1999).

[0003] Histones constitute a highly conserved family of proteins (H3, H4, H2A, H2B, H1) which are the major components of eukaryotic chromatin structure. Histones compact genomic DNA into basic repeating structural units, the nucleosomes. In addition to their DNA packaging function, histones have been proven to be integral components of the molecular machinery that regulates gene expression.

[0004] Post-translational modifications of histone N-termini, particularly of H4 and H3, are well documented and have functionally been characterised as changes in acetylation (Grunstein, 1998; Turner, 1998; Strahl and Allis, 2000), phosphorylation (Wei *et al.*, 1999) and, most recently, methylation (Chen *et al.*, 1999; Strahl *et al.*, 1999). In contrast to the large number of described histone acetyltransferases (HATs) and histone deacetylases (HDACs), genes encoding enzymatic activities that regulate phosphorylation (Sassone-Corsi *et al.*, 1999) or methylation (Chen *et al.*, 1999) of histone N-termini are only beginning to be identified. Moreover, the interdependence of the different histone tail modifications for the integration of transcriptional output or higher-order chromatin organisation is currently not understood.

[0005] Overall, there is increasing evidence that the regulation of normal and aberrant cellular proliferation is not only affected on the transcriptional level, but that also a higher level of regulation is involved, i.e. the organisation of chromatin structure through the modification of histone molecules. The determination of the proteins and the molecular mechanisms involved in histone modification will contribute to the understanding of the cellular proliferation program and will thus shed light on the mechanisms involved in aberrant proliferation occurring in tumour formation and progression (Jacobson and Pillus, 1999).

[0006] Genetic screens for suppressors of position effect variegation (PEV) in *Drosophila* (Reuter and Spierer, 1992) and *S.pombe* (Allshire *et al.*, 1995) have identified a subfamily of approximately 30-40 loci which are referred to as *Su(var)*-group (Wallrath, 1998) genes. Interestingly, several histone deacetylases (De Rubertis *et al.*, 1996), protein phosphatase type 1 (Baksa *et al.*, 1993) and S-adenosyl methionine synthetase (Larsson *et al.*, 1996) have been classified as *Su(var)s*. In contrast, *Su(var)2-5* (which is allelic to *HP1*) (Eissenberg *et al.*, 1992), *Su(var)3-7* (Cléard *et al.*, 1997) and *Su(var)3-9* (Tschiersch *et al.*, 1994; Schotta and Reuter, 2000) encode heterochromatin-associated proteins. *Su(var)* gene function thus suggests a model, in which modifications at the nucleosomal level may initiate the formation of defined chromosomal subdomains that are then stabilised and propagated by heterochromatic SU(VAR) proteins (Henikoff, 1997).

[0007] *Su(var)3-9* is dominant over most PEV modifier mutations (Tschiersch *et al.*, 1994), and mutants in the corresponding *S.pombe clr4* gene (Ivanova *et al.*, 1998) disrupt heterochromatin association of other modifying factors and result in chromosome segregation defects (Ekwall *et al.*, 1996). Recently, human (*SUV39H1*) and murine (*Suv39h1* and *Suv39h2*) *Su(var)3-9* homologues have been isolated (Aagaard *et al.*, 1999). It has been shown that they encode heterochromatic proteins which associate with mammalian HP1 (Aagaard *et al.*, 1999). The SU(VAR)3-9 protein family combines two of the most evolutionarily conserved domains of 'chromatin regulators': the chromo (Aasland and Stewart, 1995; Koonin *et al.*, 1995) and the SET (Tschiersch *et al.*, 1994; Jenuwein *et al.*, 1998) domain. Whereas the 60 amino acids chromo domain represents an ancient histone-like fold (Ball *et al.*, 1997) that directs eu- or heterochromatic localisations (Platero *et al.*, 1995), the molecular role of the 130 amino acids SET domain has remained enigmatic. Overexpression studies with human SUV39H1 mutants indicated a dominant interference with higher-order chromatin organisation that, surprisingly, suggested a functional relationship between the SET domain and the distribution of phosphorylated (at serine 10) H3 (Melcher *et al.*, 2000). The experiments of the present invention show that mammalian SU(VAR)3-9 related proteins (human SUV39H1, murine *Suv39h1* and murine *Suv39h2*) are SET domain-dependent H3-specific histone methyltransferases which selectively methylate lysine 9 ("K9") of the H3 N-terminus. Methylation of K9 negatively regulates phosphorylation of serine 10 and reveals a 'histone code' that appears intrinsically linked to the organisation of higher-order chromatin. (In the following, histone methyltransferases are termed "HMTases" or, more generally, "MTases").

[0008] It was an object of the invention to gain further insight in the molecular pathways leading to histone modifications and higher-order chromatin organisation in order to harness these findings as a target for interfering with aberrant gene expression and genomic instability through chromosome missegregation and thus provide new cancer therapies.

[0009] In particular, it was an object of the invention to investigate the function of members of the SU(VAR)3-9 protein

family with the view to develop novel strategies to affect higher-order chromatin dependent chromosome stability. Such strategies can be employed in therapies for the treatment of conditions in which aberrant gene expression and genomic instability through chromosome missegregation are causally involved. (The term "chromosome stability" implies successful segregation of chromosomes resulting in the maintenance of a stable karyotype).

[0010] To solve the problem underlying the present invention, in a first step bioinformatic techniques were applied. Using the SET domains of the SU(VAR)3-9 protein family as a starting alignment, significant sequence and secondary structure similarities (see Methods) to six plant protein methyltransferases were detected.

[0011] To investigate whether the SET domain of human SUV39H1 has enzymatic activity, histones were tested as possible substrates for *in vitro* methylation. The obtained results demonstrate that SUV39H1 harbours an intrinsic histone methyltransferase activity and suggest that this HMTase activity resides in the C-terminal SET domain.

[0012] Using recombinant proteins, both murine GST-Suv39h1(82-412) and the corresponding human SUV39H1 fusion protein [GST-SUV39H1(82-412)] were shown to be catalytically active. Short internal deletions were introduced into the two conserved regions of the SET domain core in GST-SUV39H1(82-412), and additional mutants lacking the C-terminal tail (Δ C-tail) or the SET-associated cysteine-rich region (Δ cys) were generated. All mutant proteins failed to demonstrate HMTase activity.

[0013] Although these results suggest a significant contribution by the cysteine-rich regions, their apparent absence in the plant methyltransferases does not prevent catalytic activity. To investigate enzyme function of the SET domain in more detail, point mutations were introduced into the most highly conserved motif. *In vitro* HMTase assays indicated that all point mutations, with the exception of one, abolished enzymatic activity. Surprisingly, the latter mutation resulted in an hyperactive enzyme with approximately 20-fold increased activity. The data obtained define the $_{320}\text{H}\phi\text{NHSC}_{326}$ motif in the SET domain as an important catalytic site.

[0014] Because the SET domain is one of the most conserved protein motifs in chromatin regulators (Stassen *et al.*, 1995; Jenuwein *et al.*, 1998), it was next analysed whether SU(VAR)3-9 family members or other SET domain proteins contain HMTase activity. GST-fusion products of the extended SET domains of *S.pombe* CLR4 (Ivanova *et al.*, 1998), human EZH2 (Lable *et al.*, 1997) and human HRX (Tkachuk *et al.*, 1992) were generated that would correspond to GST-SUV39H1 (82-412). Interestingly,

GST-CLR4(127-490) displayed pronounced HMTase activity at three- to five-fold increased levels as compared to the recombinant SUV39H1 product, consistent with CLR4 carrying an arginine at the hyperactive position. The results obtained from this analysis show, in agreement with the mutational analysis of SUV39H1, that HMTase activity towards free histones appears to require the combination of the SET domain with adjacent cysteine-rich regions, which is a quality found in only a restricted number of SET domain containing proteins.

[0015] These experiments indicated that the HMTase activity of mammalian SU(VAR)3-9 related proteins is selective for histone H3 under the chosen assay conditions. To examine this finding in more detail, *in vitro* methylation reactions were performed with individual histones. It could be shown that H3 is specifically methylated by GST-Suv39h1(82-412), whereas no signals are detected with H2A, H2B or H4. Methylation of H3 has been shown to occur predominantly at lysine 4 in a wide range of organisms, as well as at lysine 9 in HeLa cells, although the responsible HMTase(s) have yet to be defined (Strahl *et al.*, 1999). To investigate the site utilisation profile of Suv39h1, unmodified peptides comprising the wild-type H3 N-terminus and a mutant K9L peptide were tested as substrates. Additionally, insulin and peptides comprising the N-termini of CENP-A (Sullivan *et al.*, 1994), macroH2A (Pehrson and Fried, 1992) were included. These *in vitro* assays revealed selective methylation of the wild-type H3 peptide. The data obtained also suggested that the H3 N-terminus is a preferred residue for Suv39h1-dependent HMTase activity.

[0016] To more definitively determine this site preference, the wild-type H3 N-terminal peptide was *in vitro* methylated by GST-Suv39h1 (82-412), using S-adenosyl-[methyl- ^3H]-L-methionine. The labelled peptide, purified by reverse-phase HPLC, was then directly microsequenced, and

^3H -incorporation associated with each individual amino acid was analysed. The results confirmed selective transfer of methyl-label to lysine 9, demonstrating that Suv39h1 is a highly site-specific HMTase for the H3 N-terminus *in vitro*.

[0017] Murine *Suv39h* genes are encoded by 2 loci, *Suv39h1* and *Suv39h2*. To investigate the *in vivo* significance of *Suv39h* function and *Suv39h* dependent K9 H3 methylation, mouse strains deficient for both *Suv39h1* and *Suv39h2* were generated. *Suv39h1* and *Suv39h2* deficient strains were intercrossed to produce *Suv39h* double deficient mice. Double mutant mice were born in sub-Mendelian ratios. Some double null embryos exhibited severe growth retardation and exencephaly. In addition surviving double mutants were growth retarded, suggesting a role for *Suv39h* in cell proliferation.

[0018] In order to determine whether the embryonic phenotypes in *Suv39h* null mice can be attributed to mitotic defects, PMEFs (primary mouse embryonic fibroblasts) derived from *Suv39h* double mice were analysed. *Suv39h* double null PMEFs display a reduced G1-index and an increased proportion of cells with aberrant nuclear morphologies, reminiscent of division defects during mitosis. Furthermore, double null cells also show genomic instabilities and readily become aneuploid. The severity of these aneuploidies increases with higher passage numbers. The inability of *Suv39h* double null cells to maintain a stable karyotype may underlie the *Suv39h* embryonic phenotype.

[0019] Phosphorylation at serine 10 in the N-terminal tail of H3 (phosH3) has been shown to be required for condensation and subsequent segregation of chromosomes (Wei *et al.*, 1999). During the cell cycle, phosH3 initiates within pericentric heterochromatin in late G2 and then progresses along the entire chromosomes during mitosis (Hendzel *et al.*, 1997). It was found that in wild-type PMEFs, approximately 7% of the cells stain positive for the characteristic, heterochromatin-associated phosH3 foci. In contrast, this number is increased by a factor of about 3-fold in *Suv39h* double null PMEFs. This result suggested that the overall levels of phosH3 may be enhanced in *Suv39h* double null PMEFs. This was confirmed biochemically. Together, the obtained data are most consistent with a model in which *Suv39h*-mediated methylation of lysine 9 in H3 negatively regulates phosphorylation of serine 10.

[0020] Together, these data clearly demonstrate crucial roles for *Suv39h* during cell division. Loss of *Suv39h* function impairs K9 histone H3 methylation and induces defective cell division resulting in genome instabilities. Segregation defects/genome instability underlies the aetiology of many human cancers (Lengauer *et al.*, 1997) and are often a prerequisite for tumour progression. These observations make *Suv39h* an excellent candidate for novel therapeutic approaches for tumour therapies.

[0021] The results obtained in the experiments of the present invention show that members of the SU(VAR)3-9 protein family have HMTase activity which identifies them as novel targets for the therapy of proliferative disorders, in particular cancer.

[0022] The identification of members of the SU(VAR)3-9 protein family, exemplified by human SUV39H1, murine *Suv39h1* and murine *Suv39h2*, as K9 specific histone H3 MTases is the prerequisite for designing assay methods that allow for finding compounds altering, in particular interfering with, higher order chromatin dependent chromosome stability, which is the basis for novel therapeutic approaches.

[0023] The present invention relates to a method for identifying a compound that alters higher order chromatin dependent chromosome stability, said method comprising incubating a substrate for a methyltransferase, in the presence of a methyl donor, with a MTase with *Suv39h*-like MTase activity, in the presence or absence of a test compound and determining whether the compound modulates the MTase activity.

[0024] The group of MTases with *Suv39h*-like MTase activity (in the following also termed "*Suv39h*-like MTases") encompasses enzymes which display K9 histone H3 MTase activity or methyltransferase activity for other yet to be identified substrates.

[0025] The term "K9 histone H3" is not limited to the human SUV39H or mouse *Suv39h* substrate (i.e. the methylation site at lysine 9 of histone H3), but is meant to encompass any substrate of the histone or histone variant-type of protein the methylation of which results in the below-defined epigenetic signal.

[0026] Additional members of the group of MTases can be identified by bioinformatic/biochemical techniques and tested biochemically. By way of example, in a first step, searching data bases for similarities, as described in Example 1. Next, an identified candidate can be verified as a MTase with *Suv39h*-like MTase activity in biochemical assays similar to or identical with those described in the Examples.

[0027] This group of *Suv39h*-like MTases also encompasses MTases with specificities for other histone H3 residues than K9 or for substrates other than histone H3, which are, like the *Suv39h* K9 histone H3 HMTase activity observed in the present invention, required for higher order chromatin dependent chromosome stability. This epigenetic signal may be a consequence of histone methylation at lysine 9 on H3 alone; however, it cannot be excluded that MTase activity on undefined substrates or a combination of substrate methylation and other covalent modifications, such as phosphorylation or acetylation, at other histone residues are involved.

[0028] In the experiments of the present invention, *Suv39h* variants with point mutations in the SET domain were shown to confer hyperactive HMTase activity to the protein, these *Suv39h* variants may be advantageously used in the method of the invention.

[0029] In a preferred embodiment, the MTase is mouse *Suv39h1* or *Suv39h2*, most preferably, the MTase is human SUV39H1 or SUV39H2.

[0030] Since it has been shown in the present invention that recombinant *Suv39h* retains HMTase activity, most preferably, a recombinant MTase is employed. *Suv39h* or *Suv39h* variants can be produced recombinantly according to standard methods by expression in suitable hosts, e.g. bacteria, yeast, insect or eucaryotic cells and purified, e.g. on glutathioneagarose columns if it has been tagged with GST.

[0031] The *Suv39h1* and SUV39H1 cDNA sequences are known from the literature (Aagaard *et al.*, 1999), the *Suv39h2* cDNA sequence is shown in SEQ ID NO: 1; the human SUV39H2 cDNA is defined by the ESTs as shown in SEQ ID NO: 3 - 6.

[0032] In the case of testing the compounds for their effect on *Suv39h* activity, the assay comprises, as its essential features, incubating a histone H3 protein or histone H3 N-terminal fragment including K9, a methyl donor, S-adenosyl-L-Methionine with a preparation containing a *Suv39h* MTase activity and determining MTase activity in the presence or absence of a test substance.

[0033] MTase substrates useful in the method of the invention may be those equivalent to or mimicking the naturally occurring substrates, e.g. biochemically purified histone H3, recombinantly produced histone H3, or a histone H3 pep-

tide that contains the K9 methylation site, or other yet to be identified proteins which act as substrates for Suv39h MTases. Novel Suv39h substrates can be identified by bioinformatic and biochemical techniques and tested using the biochemical assays described in the Examples of the present invention. For example, novel Suv39h substrates can be identified by co-immunoprecipitation techniques. Suv39h proteins or tagged versions of Suv39h proteins can be immunoprecipitated with specific antisera and interacting proteins identified by mass spectroscopy techniques. A yeast two hybrid screen using Suv39h proteins or portions of Suv39h proteins as a bait can also be employed to identify novel interacting protein from a variety of cDNA libraries.

[0034] In a preferred embodiment, the histone H3 fragment

ARTKQTARKSTGGKAPRKQL (SEQ ID NO:7) is employed. Alternatively, a modified peptide may be used for which the MTase has increased affinity/activity. Such peptides can be designed by exchanging and/or adding and/or deleting amino acids and testing the substrate in serial experiments for MTase affinity/activity.

[0035] The methyl donor preferably carries a detectable label, e.g. a radioactive or a chromogenic label, which can be quantified upon transfer to the substrate.

[0036] Preferably, the methyl donor is radio-labelled methionine or S-adenosyl-L-methionine.

[0037] Alternatively to using a labelled methyl donor, the substrate, upon methylation by the enzyme, is used to serve as an epitope which can be recognised by a specific antibody and hence be quantified by standard immunoassay techniques, e.g. ELISAs. Antibodies useful in this type of assay can be obtained by using the methylated substrate, preferably a small peptide, e.g. the peptide with the sequence shown in SEQ ID NO:7, as an antigen and obtaining polyclonal or monoclonal antibodies according to standard techniques.

[0038] For small scale applications, the screening method can be based on an assay as described in Example 2, 3 or 4.

[0039] In a preferred embodiment, the method of the invention is performed on a high-throughput scale. For this embodiment, the major assay components, in particular Suv39h, are employed in recombinant form.

[0040] For the high throughput format, the screening methods of the invention to identify MTase inhibitors, are carried out according to standard assay procedures. Such assays are based on the catalytic transfer, mediated by Suv39h or a Suv39h variant, of a methyl group from a chromogenic substrate to a histone H3 peptide. To achieve this, the substrate, e.g. histone H3 or a variant or fragment thereof, is immobilised on a carrier, usually a microtiter plate, and incubated with recombinant Suv39h or a Suv39h variant and a methyl donor. The methyl donor preferably carries a chromogenic or radioactive label. Upon transfer of the methyl group to the substrate by Suv39h, in the case of a chromogenic reagent, the methyl donor changes colour which can be quantified. In the case of using a radioactive methyl donor, the methyl group is transferred to the substrate and can be quantified.

[0041] Alternatively, the methylation of the substrate can be quantified by ELISA using an antibody specific for the methylated substrate. If a test substance is an inhibitor of the MTase activity, there will be, depending on the detection system and depending on whether the test substance has an inhibiting or an activating effect, a decrease or an increase in the detectable signal.

[0042] In the high-throughput format, compounds with a modulating effect Suv39h MTase activity can be identified by screening test substances from compound libraries according to known assay principles, e.g. in an automated system on microtiter plates.

[0043] By providing a method to identify compounds which exert their effect by directly modulating, in particular by inhibiting, a Suv39h-like MTase, the present invention provides a basis for inhibiting the proliferation of rapidly dividing animal cells, in particular tumour cells.

[0044] The compounds identified in the above methods, which are also subject of the invention, have the ability to interfere with chromosome stability and high fidelity chromosome segregation by modulating the MTase activity of Suv39h.

[0045] In a preferred embodiment, the compounds of the invention are inhibitors of Suv39h HMTase activity.

[0046] Preferably, the compounds are specific modulators of Suv39h.

[0047] The present invention also relates to compounds, which act as modulators of a Suv39h-like MTase activity, in particular modulators of Suv39h, for use in human therapy, in particular cancer therapy.

[0048] Compounds inhibiting Suv39h HMTase activity result in decreased genome stability and can be used in therapy, optionally in combination with other genome destabilising agents, to target highly proliferative tumour cells. Likewise, agents which enhance Suv39h HMTase activity can be used to stabilise the genome of inherently unstable cells rendering them less prone to acquiring proliferation promoting mutations. A model for *Suv39h* function and effects of inhibition or enhancement of Suv39h enzymes is shown in Figure 8.

[0049] Compounds inhibiting Suv39h HMTase activity result in decreased genome stability and can be used in therapy, optionally in combination with other genome destabilising agents, to target highly proliferative tumour cells. Likewise, agents which enhance Suv39h HMTase activity can be used to stabilise the genome of inherently unstable cells, rendering them less prone to acquiring proliferation promoting mutations.

[0050] The efficacy of compounds identified as Suv39h modulators can be tested for *in vivo* efficacy in mammalian

cells with *Suv39h* double null cells serving as a positive control. Effective compounds should interfere with chromosome stability and segregation, which can be measured by karyotyping, e.g. by analysing the DNA content by FACS or standard cytological techniques. Substances whose potential for therapeutic use has been confirmed in such secondary screens can be further tested for their effect on tumour cells. To test the inhibition of tumour cell proliferation, primary human tumour cells are incubated with the compound identified in the screen and the inhibition of tumour cell proliferation is tested by conventional methods, e.g. bromo-desoxy-uridine or ^3H thymidine incorporation. Compounds that exhibit an anti-proliferative effect in these assays may be further tested in tumour animal models and used for the therapy of tumours.

[0051] Toxicity and therapeutic efficacy of the compounds identified as drug candidates by the method of the invention can be determined by standard pharmaceutical procedures, which include conducting cell culture and animal experiments to determine the IC_{50} , LD_{50} , the ED_{50} . The data obtained are used for determining the human dose range, which will also depend on the dosage form (tablets, capsules, aerosol sprays, ampules, etc.) and the administration route (oral, buccal, nasal, paterental or rectal). A pharmaceutical composition containing the compound as the active ingredient can be formulated in conventional manner using one or more physiologically active carriers and excipients. Methods for making such formulations can be found in manuals, e.g. "Remington Pharmaceutical Sciences".

[0052] As *Suv39h* is required to maintain a stable karyotype, it can be considered as a tumour suppressor gene. If *Suv39h* mutations prove to be a factor underlying cellular transformation events - which can be confirmed by analysis of *Suv39h* double deficient mice for tumour development and progression -, it can be expected that the re-introduction of a wild type *Suv39h* gene by gene therapy results in increased genomic stability delaying or inhibiting cancer progression.

[0053] For gene therapy, the *Suv39h* DNA molecule may be administered, preferably contained on a plasmid in recombinant form, directly or as part of a recombinant virus or bacterium. In principle, any method of gene therapy may be used for applying *Suv39h* recombinant DNA, both *in vivo* and *ex vivo*.

[0054] Examples of *in vivo* administration are the direct injection of "naked" DNA, either by intramuscular route or using gene guns. Examples of recombinant organisms are vaccinia virus or adenovirus. Moreover, synthetic carriers for nucleic acids such as cationic lipids, microspheres, micropellets or liposomes may be used for *in vivo* administration of nucleic acid molecules coding for the *Suv39h* polypeptide.

[0055] Since it has been shown in the present invention that *Suv39h* mediates dynamic transitions in higher-order mammalian chromatin in part through its intrinsic HMTase activity, K9 methylation of histone H3 (K9-Me) represents an important epigenetic imprint for chromosome dynamics during cell division. Hence, antibodies specific for K9-Me can be used to screen cells/patients for heterochromatin based genome instabilities. In essence, K9-methylation specific anti-sera can be used as a diagnostic tool for several potential human diseases.

Brief description of figures:

[0056]

Figure 1: HMTase activity of transfected and recombinant SUV39H1 and *Suv39h1* proteins.

Figure 2: Specific HMTase activity of the SET domain of mammalian SU(VAR)3-9 related proteins.

Figure 3: Lysine 9 of the H3 N-terminus is the major site for *in vitro* methylation by recombinant *Suv39h1*.

Figure 4: Targeting *Suv39h1* and *Suv39h2* in the mouse germline.

Figure 5: Analysis of *Suv39h* double null PMEFs.

Figure 6: Genomic instabilities in *Suv39h* double null PMEFs.

Figure 7: Increased phosH3 phosphorylation in *Suv39h* double null PMEFs.

Figure 8: Model for *Suv39h* function.

Materials and Methods

[0057]

a) Sequence alignments and secondary structure predictions

The SET domains of human SUV39H1 (Aagaard *et al.*, 1999), *Drosophila* SU(VAR)3-9 (Tschiersch *et al.*, 1994) and *S.pombe* CLR4 (Ivanova *et al.*, 1998) were used as a multiple starting alignment for database similarity searches using Profile (Birney *et al.*, 1996), hidden Markov (Eddy, 1998) and position-specific iterative BLAST (Altschul *et al.*, 1997) methods (representative listings are available from the SET domain page of the SMART WWW-server (Schultz *et al.*, 2000). These searches revealed significant similarities to six plant proteins (accession numbers Q43088, 065218, P94026, 080013, AAC29137 and AC007576_12) described as putative lysine N-methyltransferases. For example, a PSI-BLAST search with the *S.pombe* hypothetical protein SPAC3c7.09 as query identified these plant sequences and well-known SET domain sequences within ten rounds using an E-value inclusion threshold of 0.001. The same search also revealed the presence of a SET domain in YHR109w (which is known to encode a cytochrome c MTase (Martzen *et al.*, 1999)) within three rounds. Consensus secondary structures were predicted by described algorithms (Frishman and Argos, 1997).

b) Epitope-tagged SUV39H1 proteins in HeLa cells

The HeLa cell lines overexpressing full-length (myc)3-SUV39H1 (aa 3-412) or (myc)3-Nchromo (aa 3-118) have been described (Aagaard *et al.*, 1999; Melcher *et al.*, 2000). Nuclear extracts were immunoprecipitated with anti-myc antibody beads (Aagaard *et al.*, 1999), and approximately 1-3 µg of matrix-bound (myc)3-tagged SUV39H1 proteins were used for *in vitro* HMTase assays.

c) Generation and purification of GST-fusion proteins

The GST-Suv1(82-412) product expressed from the pGEX-2T vector (Pharmacia) as a glutathione-S-transferase (GST) fusion protein has been described (Aagaard *et al.*, 1999). Additional GST constructs were generated by transferring BamHI-EcoRI PCR amplicons into pGEX-2T, encoding in-frame fusions for Suv39h1(7-221), SUV39H1(82-412), SUV39H1(82-378) ΔC-tail, SUV39H1(255-412) Δcys, Suv39h2(157-477) (O'Carroll *et al.*, submitted), CLR4(127-490) (Ivanova *et al.*, 1998), EZH2(382-747) (Lable *et al.*, 1997) and HRX(3643-3969) (Tkachuk *et al.*, 1992). Short internal deletions (ΔNHSCDPN₃₂₃₋₃₂₉ and ΔGEELTFDY₃₅₈₋₃₆₅) or point mutations within the ₃₂₀HφNHSC₃₂₆-motif were directly engineered in the GST-SUV39H1(82-412) plasmid by double PCR mutagenesis. All constructs were confirmed by sequencing.

Recombinant proteins were expressed in 1l cultures of *E.coli* strain BL21 and solubilized in 10 ml RIPA buffer [(20 mM Tris pH 7.5, 500 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate) containing a full set of protease inhibitors (Boehringer Mannheim) and lysozyme (5 mg/ml; Sigma)] by freeze-thawing in liquid N₂, followed by sonication. Soluble proteins were cleared by centrifugation, purified with 800 µl glutathione Sepharose beads (Pharmacia) and washed twice in RIPA buffer. Protein concentration was determined by Coomassie staining of SDS-PAGE gels. Matrix-bound fusion proteins were used immediately for *in vitro* HMTase assays or stored at 4°C.

d) *In vitro* histone methyltransferase (HMTase) assay

In vitro HMTase reactions were modified based on described protocols (Strahl *et al.*, 1999) and carried out in a volume of 50 µl of methylase activity buffer (MAB: 50 mM Tris pH 8.5, 20 mM KCl, 10 mM MgCl₂, 10 mM β-ME, 250 mM sucrose), containing 10 µg of free histones (mixture of H1, H3, H2B, H2A and H4; Boehringer Mannheim) as substrates and 300 nCi S-adenosyl-[methyl-¹⁴C]-L-methionine (25 µCi/ml) (Amersham) as methyl donor. 10 µg of matrix-bound GST-fusion proteins were routinely used to assay for HMTase activity. After incubation for 60 min. at 37°C, reactions were stopped by boiling in SDS loading buffer, and proteins were separated by 15% or 18% SDS-PAGE and visualised by Coomassie staining and fluorography.

HMTase assays with individual histones (Boehringer Mannheim), insulin (Sigma) or N-terminal peptides were performed with 5 µg of substrate. The following peptides were used: wild-type N-terminus of human histone H3 (ARTKQTARKSTG-GKAPRKQL) (SEQ ID NO:7) and mutant peptide which changes lysine 9 (bold) to leucine; N-terminus of human CENP-A (MGPRRRSRKPEAPRRRSPSP) (SEQ ID NO:8) (Sullivan *et al.*, 1994); N-terminus of rat macro-H2A (MSSRGGKKKSTKTSRSKAG) (SEQ ID NO:9) (Pehrson and Fried, 1992).

Peptide microsequencing of the *in vitro* methylated wild-type H3 N-terminal peptide and determination of ³H-incorporation of individual amino acids by scintillation counting was done as described (Strahl *et al.*, 1999).

e) Generation of Suv39h1 and Suv39h2 deficient mice by gene targeting

Suv39h1 maps to the X-chromosome. The cloned partial *Suv39h1* genomic locus (O'Carroll *et al.*, submitted) was used to generate a targeting construct. A 1.2kb Pfu PCR amplicon, generated with the primers gM3-9(SII) and gM3-9(RI), was used as a short arm of homology and cloned in frame with the nls-lacZ gene of the pGNA-T vector. This places the first 3 amino acids of exon 2 in frame with the nls-lacZ gene generating a fusion protein of the first 8 amino acids of Suv39h1 and lacZ from the targeted locus. A 5.4kb SacI (filled in) fragment from *Suv39h1* genomic subclone gSuv39h1 #18 was used as a long arm of homology.

Suv39h2 is autosomal and maps to chromosome 2. The cloned partial *Suv39h2* genomic locus (O'Carroll *et*

al., submitted) was used to generate a targeting construct. A 1.4 kb Pfu PCR amplicon, generated with the primers Suv2SII and Suv2RI, was used as a short arm of homology and cloned in frame with the nls-lacZ gene of the pGNA-T vector. This places the first 113 amino acids of exon 2 in frame with the nls-lacZ gene generating a fusion protein of the first 113 amino acids of Suv39h2 and lacZ from the targeted locus. A 4.9kb MluI/ApaI (filled in) fragment from *Suv39h2* genomic subclone *gSuv39h2#28* was used as long arm of homology to inactivate the locus.

These constructs were linearised with NotI, electroporated into R1 ES cells (*Suv39h1*) and E14.1 ES cells (*Suv39h2*), ES cells were put under G418 selection and G418 resistant colonies screened for homologous recombination by PCR and Southern blot analysis. Targeted feeder dependent ES cell clones were injected into blastocysts of C57BL/6 mice and reimplanted into pseudopregnant females to produce chimeric offspring. Germ-line transmission was obtained after a backcross between chimeric males and C57BL/6 females. Heterozygous mice were interbred to obtain *Suv39h1* and *Suv39h2* deficient mice. *Suv39h1* and *Suv39h2* deficient mice were then interbred to generate *Suv39h* double deficient mice.

f) Generation and analysis of *Suv39h* double null primary mouse embryonic fibroblasts (PMEFs)

PMEFs were derived from day E12.5 *Suv39h* double null embryos obtained after intercrossing *Suv39h1^{-/-}Suv39h2^{-/-}* compound mutant mice. As controls, PMEFs were prepared from wild-type embryos of the same genetic background. For cell cycle profiles and growth curve analysis, passage 2 PMEFs were analyzed as described (Xu *et al.*, 1999). Staining of PMEF interphase chromatin with anti-phosphH3 (Hendzel *et al.*, 1997) antibodies was done in untreated cells. For the biochemical analysis, total nuclear extracts were precalibrated by Ponceau staining, immuno-blotted with anti-H3 (Upstate Biotechnology) and anti-phosphH3 (Hendzel *et al.*, 1997) antibodies and visualised by peroxidase staining using Enhanced ChemiLuminescence (ECL) (Amersham).

Example 1

Sequence similarity of SET domains with plant methyltransferases

[0058] Using the SET domains of the SU(VAR)3-9 protein family as a starting alignment, significant sequence and secondary structure similarities (see Methods) to six plant protein methyltransferases were detected. Although some of these plant sequences have been classified as potential histone lysine N-methyltransferases, only one had been functionally characterised but was found to lack HMTase activity (Klein and Houtz, 1995; Zheng *et al.*, 1998).

[0059] Detected were amino acid and secondary structure [β -sheet (b) or α -helix (h)] similarities of the C-terminal halves of SET domain sequences from human SUV39H1 (Aagaard *et al.*, 1999) (AF019968), murine Suv39h1 (Aagaard *et al.*, 1999) (AF019969), murine Suv39h2 (O'Carroll *et al.*, submitted), (AF149205), *Drosophila* SU(VAR)3-9 (Tschi-sch *et al.*, 1994) (P45975), a *C.elegans* SU(VAR)3-9-like ORF C15H11.5 (CAB02737), *S.pombe* CLR4 (Ivanova *et al.*, 1998) (074565), human EZH2 (Laible *et al.*, 1997) (Q15910), the human trithorax homologue HRX (Tkachuk *et al.*, 1992) (Q03164), and MTases from *Psativum* (rubisco ls-MT; Q43088) (Klein and Houtz, 1995; Zheng *et al.*, 1998) and *A.thaliana* (065218). The plant MTase sequences contain an insertion of approximately 100 amino acids in the middle of the SET domain.

Example 2

[0060] HMTase activity of transfected and recombinant SUV39H1 and Suv39h1 proteins.

[0061] To investigate whether the SET domain of human SUV39H1 has enzymatic activity, histones were tested as possible substrates for *in vitro* methylation. Using HeLa cell lines 'stably' expressing triple myc-tagged full-length SUV39H1 (aa 3-412), the ectopic protein was enriched from nuclear extracts by immunoprecipitation with anti-myc beads (see Figure 1/1A, arrowhead top panel) and probed for activity to transfer a labelled methyl group from S-adenosyl-[methyl-¹⁴C]-L-methionine to free histones according to described conditions (Strahl *et al.*, 1999). Reaction products were separated by SDS-PAGE and visualised by fluorography, indicating selective transfer of the methyl-label to H3 (Figure 1/1A, lower panel). By contrast, no signals were detected with extracts from a HeLa cell line that expresses only the N-terminal third of SUV39H1 (aa 3-118) or with extracts from HeLa control cells. To confirm that the HMTase activity is an intrinsic property of SUV39H1 and not mediated by a SUV39H1-associated factor, the *in vitro* HMTase reactions were repeated with recombinant products that were purified as GST-fusion proteins from *E.coli* (see Figure 1/2B, arrowheads top panel). For this analysis, murine Suv39h1, which is 95% identical to human SUV39H1 (Aagaard *et al.*, 1999) was used. A purified GST-product comprising aa 82-412 maintained HMTase activity (although at a reduced level as compared to transfected SUV39H1), whereas a purified GST-product comprising aa 7-221 proved negative, even at higher protein concentrations (Figure 1/2B, lower panel). These results suggest that the HMTase activity resides in the C-terminal SET domain.

[0062] In Fig. 1A, triple myc-tagged full-length human SUV39H1 (aa 3-412) or a C-terminally truncated SUV39H1

protein (aa 3-118) were immunoprecipitated from 'stably' transfected HeLa cell lines with anti-myc antibody beads and used in *in vitro* HMTase reactions with free histones as substrates and S-adenosyl-[methyl-¹⁴C]-L-methionine as methyl donor. The Coomassie stain (top panel) shows purified proteins by arrowheads and free histones by dots. Fluorography (bottom panel, Figure 1A) indicates HMTase activity of (myc)3-SUV1 (3-412).

[0063] In the experiments shown in Fig. 1B, recombinant GST-fusion proteins encoding different domains of murine Suv39h1 were used in increasing protein concentrations for *in vitro* HMTase reactions as described above.

Example 3

[0064] Definition of a catalytic motif in the SET domain of human SUV39H1.

[0065] Similar to the recombinant murine GST-Suv39h1 (82-412) product, the corresponding human SUV39H1 fusion protein [GST-SUV39H1 (82-412)] is catalytically active (see Figure 2). Short internal deletions (Δ NHSCDPN₃₂₃₋₃₂₉; Δ NHSC and Δ GEELTFDY₃₅₈₋₃₆₅; Δ GEEL) were introduced into the two conserved regions of the SET domain core in GST-SUV39H1 (82-412) and, in addition, mutants that lack the C-terminal tail (DC-tail) or the SET-associated cysteine-rich region (\square cys) were also generated. All mutant proteins failed to demonstrate HMTase activity (see Figure 2/1A). To investigate enzyme function of the SET domain in more detail, point mutations were introduced into the most highly conserved motif. *In vitro* HMTase assays indicated that all point mutations, with the exception of one, abolished enzymatic activity. Surprisingly, the latter mutation (H320R) resulted in an hyperactive enzyme with approximately 20-fold increased activity. The data obtained define the ₃₂₀H $\square\square$ NHSC₃₂₆ motif in the SET domain as an important catalytic site.

[0066] Specific HMTase activity of the SET domain of mammalian SU(VAR)3-9 related proteins.

[0067] Because the SET domain is one of the most conserved protein motifs in chromatin regulators (Stassen *et al.*, 1995; Jenuwein *et al.*, 1998), it was next analyzed whether SU(VAR)3-9 family members or other SET domain proteins contain HMTase activity. GST-fusion products of the extended SET domains of *S.pombe* CLR4 (Ivanova *et al.*, 1998), human EZH2 (Laible *et al.*, 1997) and human HRX (Tkachuk *et al.*, 1992) were generated that would correspond to GST-SUV39H1 (82-412) (see Figure 2/2B). Interestingly, GST-CLR4 (127-490) displayed pronounced HMTase activity at three- to five-fold increased levels (see Figure 2/2C) as compared to the recombinant SUV39H1 product, consistent with CLR4 carrying an arginine at the hyperactive position. By contrast, both GST-EZH2 (382-747) and GST-HRX (3643-3966) had undetectable HMTase activity towards free histones (Figure 2/2C), whereas a comparable GST product generated from the recently isolated murine *Suv39h2* gene (O'Carroll *et al.*, submitted), GST-Suv39h2 (157-477), was as active as GST-SUV39H1 (82-412). EZH2 lacks the C-terminal cysteines, and HRX does not contain the SET associated cysteine-rich region (Figure 2B). Both of these cysteine domains are present in CLR4, Suv39h2 and SUV39H1. In agreement with the mutational analysis of SUV39H1, it thus appears that HMTase activity towards free histones requires the combination of the SET domain with adjacent cysteine-rich regions, and is a quality found in only a restricted number of SET domain containing proteins.

[0068] In Fig. 2A, approximately 10 μ g of the indicated fusion proteins encoding GST-SUV1 (82-412) (= human SUV39H1) and seven SET domain mutants were used in *in vitro* HMTase reactions with free histones as outlined in Figure 1. For the hyperactive H320R mutant, only 1 μ g (10%) of the corresponding fusion product was used. Fig 2 shows a diagram representing the domain structures of CLR4, Suv39h2, SUV39H1, EZH2 and HRX proteins, with the arrowheads demarcating the N-terminal fusion to GST. Cysteine-rich regions are indicated by grey stippling.

[0069] In Fig. 2C, approximately 10 μ g of the indicated fusion proteins encoding *S.pombe* CLR4 [GST-CLR4 (127-490)], murine Suv39h2 [GST-Suv2 (157-477)], human EZH2 [GST-EZH2 (382-747)], human HRX [GST-HRX (3643-3969)] and human SUV39H1 [GST-SUV1 (82-402)] were used in *in vitro* HMTase reactions with free histones as outlined in Figure 1.

Example 4

[0070] Lysine 9 of the H3 N-terminus is the major site for *in vitro* methylation by recombinant Suv39h1.

[0071] The above Examples indicated that the HMTase activity of mammalian SU(VAR)3-9 related proteins is selective for H3 under the chosen assay conditions. To examine this finding in more detail, *in vitro* methylation reactions were performed with individual histones, using GST-Suv39h1 (82-412) as an enzyme. As shown in Figure 3/1A, H3 is specifically methylated by GST-Suv39h1 (82-412), whereas no signals are detected with H2A, H2B or H4. A weak signal is present if H1 was used as the sole substrate; the significance of H1 methylation remains to be determined. Methylation of H3 has been shown to occur predominantly at lysine 4 in a wide range of organisms, as well as at lysine 9 in HeLa cells, although the responsible HMTase(s) have yet to be defined (Strahl *et al.*, 1999). To investigate the site utilisation profile of Suv39h1, unmodified peptides comprising the wild-type H3 N-terminus (aa 1-20) and a mutant K9L peptide, changing lysine 9 to leucine were tested as substrates. Additionally, insulin and peptides comprising the N-termini of CENP-A (Sullivan *et al.*, 1994) and macroH2A (Pehrson and Fried, 1992) were included. Peptides were *in vitro* methylated by GST-Suv39h1 (82-412), and reaction products were separated by high percentage SDS-PAGE and

visualised by fluorography. These *in vitro* assays revealed selective methylation of the wild-type H3 peptide, whereas no signals were detected with the CENP-A or macroH2A peptides, or with insulin (see Figure 3/2B). Importantly, the mutated H3 (K9L) peptide was not a substrate, suggesting that lysine 9 of the H3 N-terminus is a preferred residue for Suv39h1-dependent HMTase activity.

[0072] To more definitively determine this site preference, the wild-type H3 N-terminal peptide was *in vitro* methylated by GST-Suv39h1(82-412), using S-adenosyl-[methyl-³H]-L-methionine. The labelled peptide, purified by reverse-phase HPLC, was then directly microsequenced, and ³H-incorporation associated with each individual amino acid was analysed by scintillation counting. The results confirmed selective transfer of methyl-label to lysine 9 (see Figure 3/2C), demonstrating that Suv39h1 is a highly site-specific HMTase for the H3 N-terminus *in vitro*.

[0073] In Fig. 3A, approximately 10 µg of murine GST-Suv39h1(82-412) were used in *in vitro* HMTase reactions with individual histones as outlined in Figure 1.

[0074] Fig 3B shows the results of *in vitro* methylation assays using GST-Suv39h1(82-412) as enzyme and the indicated N-terminal peptides of wild-type H3, mutated H3 (K9L), CENP-A, macroH2A or insulin as substrates.

[0075] Fig 3C shows the result of automated sequencing of the wild-type H3 N-terminal peptide (aa 1-20) that had been methylated *in vitro* by recombinant GST-Suv39h1(82-412). Displayed is the ³H-incorporation of individual amino acids identified at each successive round of microsequencing.

Example 5

[0076] Targeting the *Suv39h1* and *Suv39h2* loci in the mouse germline.

[0077] Murine *Suv39h* genes are encoded by 2 loci, *Suv39h1* and *Suv39h2* (O'Carroll *et al.*, submitted). To investigate the *in vivo* significance of *Suv39h* function and *Suv39h* dependent K9 H3 methylation, mouse strains deficient for both *Suv39h1* and *Suv39h2* were generated according to standard techniques. The targeting strategies are shown in Figure 4, as well as demonstrating the production of null alleles for both *Suv39h1* and *Suv39h2*. Mutation of either gene results in viable and fertile mice as a consequence of functional redundancy between both loci. Therefore, *Suv39h1* and *Suv39h2* deficient strains were intercrossed to produce *Suv39h* double deficient mice.

[0078] Double mutant mice are born in sub-Mendelian ratios, approximately 20% of the expected double mutants are observed. Some double null embryos exhibit severe growth retardation and exencephaly. In addition, surviving double mutants are growth retarded, suggesting a role for *Suv39h* in cell proliferation.

[0079] Fig. 4 shows a conventional targeting strategy used to inactivate the X-linked *Suv39h1* locus. Fig. 4B shows the Northern blot analysis of *Suv39h1* from spleen (Sp), liver (Li), kidney (Kidney), and brain (Br) from wild-type and *Suv39h1* null mice. Fig. 4C shows the conventional targeting strategy used to inactivate the autosomal *Suv39h2* locus. (Bottom panel) Western blot analysis with anti-Suv39h2 antibodies on protein extracts derived from wild-type and *Suv39h2* null testis.

Example 6

[0080] Increased aneuploidies in *Suv39h* double null primary mouse fibroblasts (PMEFs).

[0081] In order to determine whether the embryonic phenotypes in *Suv39h* double null mice can be attributed to mitotic defects, PMEFs derived from *Suv39h* double mice were analysed. Cell cycle profiles of wild-type and *Suv39h* double null PMEFs indicated rather similar percentages of cells to be in S- and G2/M-phases (see Figure 5A), whereas *Suv39h* double null PMEFs display a reduced G1-index and an increased proportion of cells with aberrant nuclear morphologies, reminiscent of division defects during mitosis. For example, *Suv39h* double null PMEFs contain approximately two-fold elevated numbers of cells with micro- and polynuclei, and are further characterised by cell subpopulations with oversized nuclei or a weak definition of heterochromatin that appears in only a few unusually condensed foci (see Figure 5B). Furthermore, double null cells also show genomic instabilities and readily become aneuploid. The severity of these aneuploidies increases with higher passage numbers (see Figure 6). The inability of double null cells to maintain a stable karyotype may underlie the *Suv39h* embryonic phenotype.

[0082] Fig. 5A shows the percentages of cells in various cell cycle stages of wild-type and *Suv39h* double null PMEFs.

[0083] Fig. 5B shows representative images (left and middle) of aberrant mitoses in *Suv39h* double null PMEFs detected by β-tubulin (not shown) and DAPI staining. Also shown (right image) is a nucleus exemplifying the unusual definition of heterochromatin in a subpopulation of *Suv39h* double null PMEFs. All images were taken at a magnification of 630x.

[0084] Fig. 6A shows the DNA content profile of wild-type and *Suv39h* double null PMEFs at passage 3. Fig. 6B shows the DNA content profile of wild-type and *Suv39h* double null PMEFs at passage 8.

Example 7

[0085] Increased phosH3 phosphorylation in *Suv39h* double null PMEFs.

[0086] Phosphorylation at serine 10 in the N-terminal tail of H3 (phosH3) has been shown to be required for condensation and subsequent segregation of chromosomes (Wei *et al.*, 1999). During the cell cycle, phosH3 initiates within pericentric heterochromatin in late G2 and then progresses along the entire chromosomes during mitosis (Hendzel *et al.*, 1997). In wild-type PMEFs, approximately 7% of the cells stain positive for the characteristic, heterochromatin-associated phosH3 foci, as detected by indirect immunofluorescence with anti-phosH3 antibodies (see Figure 7A, right panel). In contrast, this number is increased by a factor of about 3-fold in *Suv39h* double null PMEFs, with approximately 22% of the cells containing phosH3-positive foci (Figure 7A, left panel), although their definition appears in many small speckles which do not always overlap with DAPI-dense material (data not shown). This result suggested that the overall levels of phosH3 may be enhanced in *Suv39h* double null PMEFs. Therefore, the relative abundance of phosH3 in precalibrated nuclear extracts was determined with anti-phosH3-specific antibodies. This quantitation indicated a significantly higher level of phosH3 in *Suv39h* double null cells as compared to wild-type controls (see Figure 7B). Together, the obtained data are most consistent with a model in which Suv39h-mediated methylation of lysine 9 in H3 negatively regulates phosphorylation of serine 10.

[0087] Fig. 7A shows an interphase chromatin staining with anti-phosH3 antibodies and CY3-conjugated secondary antibodies. DNA was counterstained with DAPI. At least 1,000 cells were counted to evaluate the percentage (indicated in the Figure) of anti-phosH3 positive cells. Fig 7B shows a quantitative Western analysis with 15 µg and 30 µg of total nuclear proteins immuno-blotted with anti-H3 and anti-phosH3 antibodies.

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[0088]

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Claims

1. A method for identifying a compound that alters higher order chromatin dependent chromosome stability, said method comprising incubating a substrate for a methyltransferase, in the presence of a methyl donor, with a methyltransferase with Suv39h-like methyltransferase activity, in the presence or absence of a test compound and determining whether the compound modulates the methyltransferase activity.
2. The method of claim 1, wherein the methyltransferase with Suv39h-like activity is murine Suv39h1 or human SUV39H1.
3. The method of claim 1, wherein the methyltransferase with Suv39h-like activity is murine Suv39h2 or human SUV39H2.
4. The method of any one of claims 1 to 3, wherein the substrate is histone H3 or N-terminal fragment thereof that contains the methylation site at lysine 9.
5. The method of claim 4, wherein the histone H3 N-terminal fragment has the amino acid sequence as set forth in SEQ ID NO:7.
6. The method of any one of claims 1 to 5, wherein the methyl donor is radio-labelled methionine or S-adenosyl-L-methionine.
7. The method of any one of claims 1 to 6, wherein the methyl donor carries a chromogenic label and the methyltransferase activity is determined by measuring the change in colour upon transfer of the methyl group to the substrate.
8. The method of any one of claims 1 to claim 6, wherein the methyl donor carries a radioactive label and the methyltransferase activity is determined by measuring the radioactivity transferred to the substrate upon transfer of the methyl group.
9. The method of any one of claims 1 to 6, wherein the methyltransferase activity is determined immunologically by quantifying the binding of an antibody specific for the methylation site to the substrate.
10. An antibody that specifically recognises methylated lysine 9 in histone H3.
11. A compound identified in a method defined in any one of claims 1 to 9 for the therapy of cancer.

Fig. 1/1

A

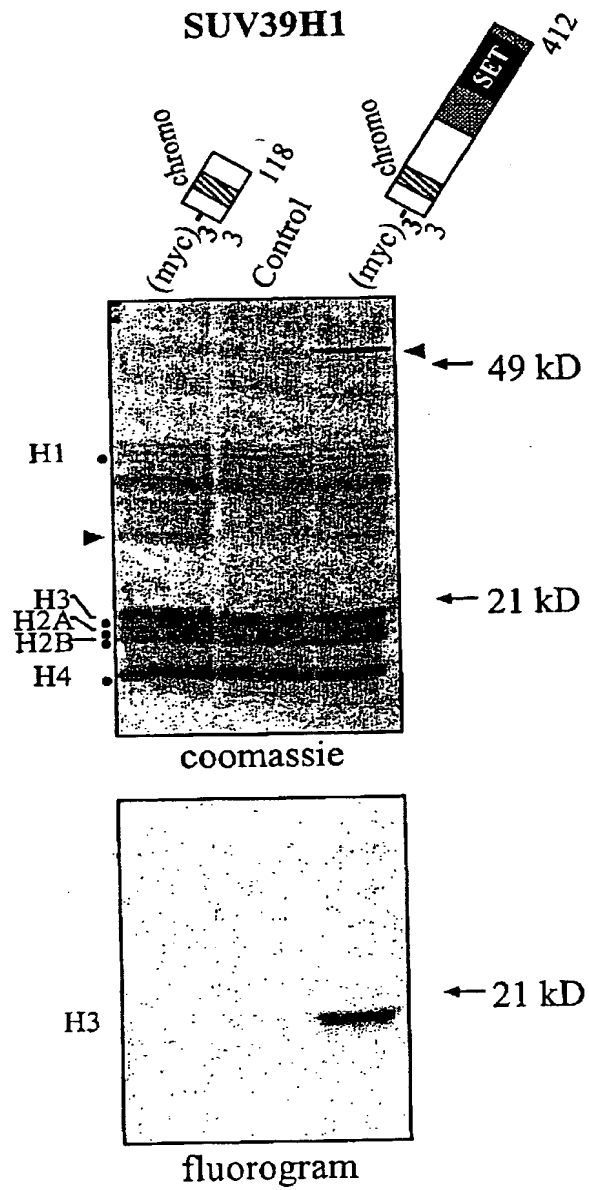


Fig. 1/2

B

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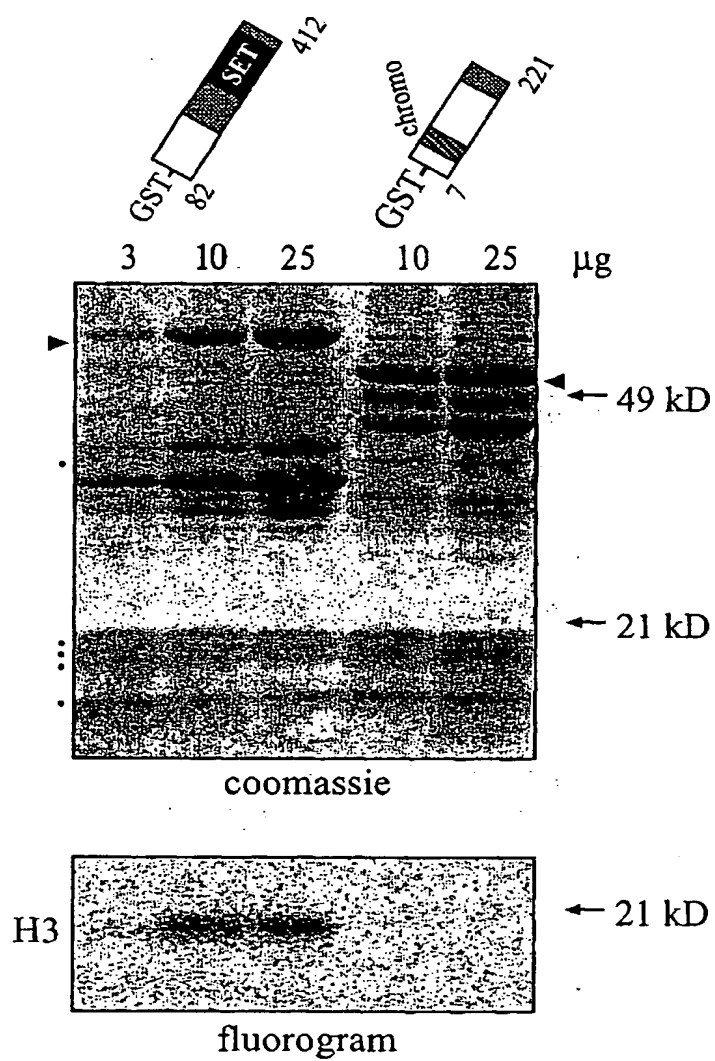


Fig. 2/1

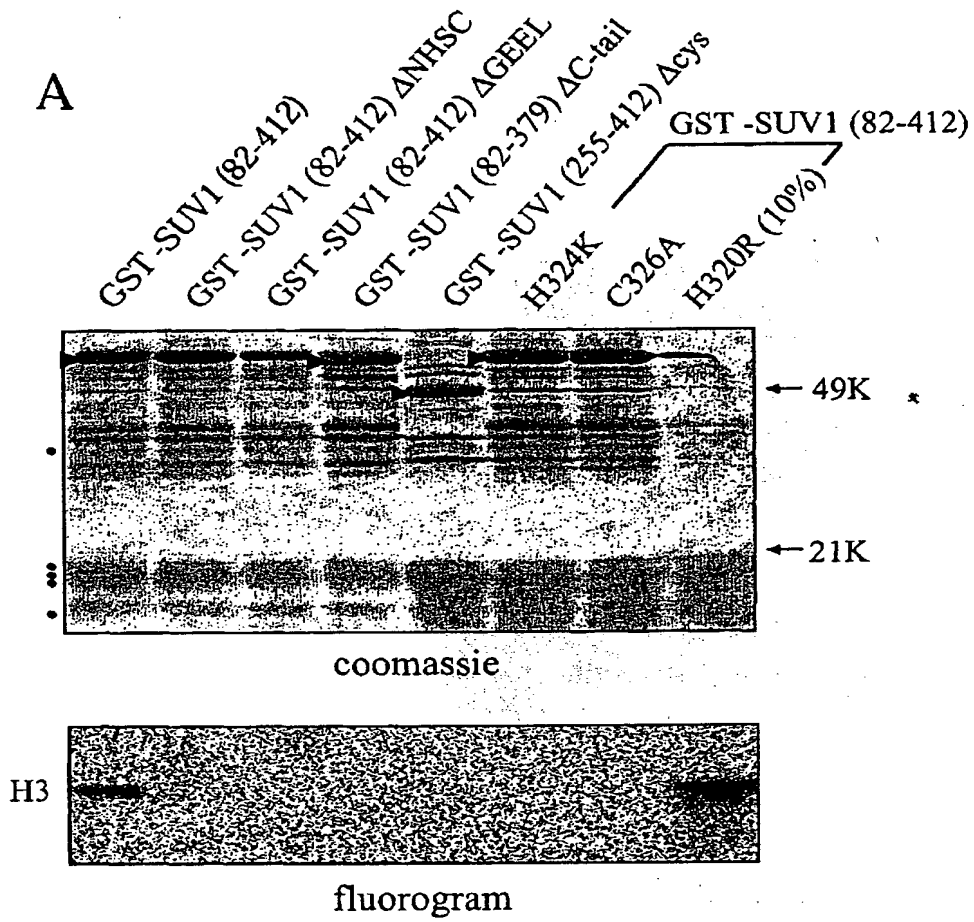
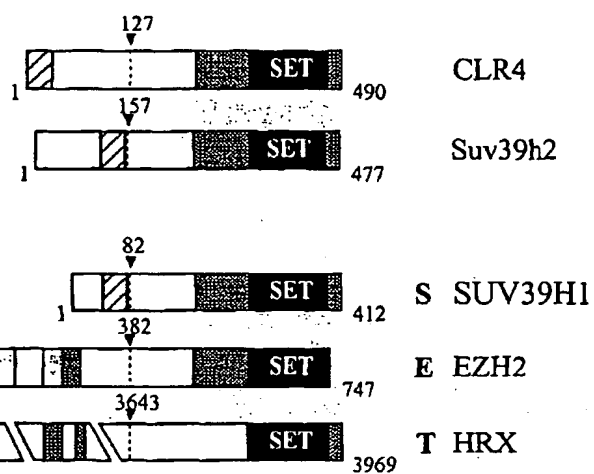


Fig. 2/2

B



C

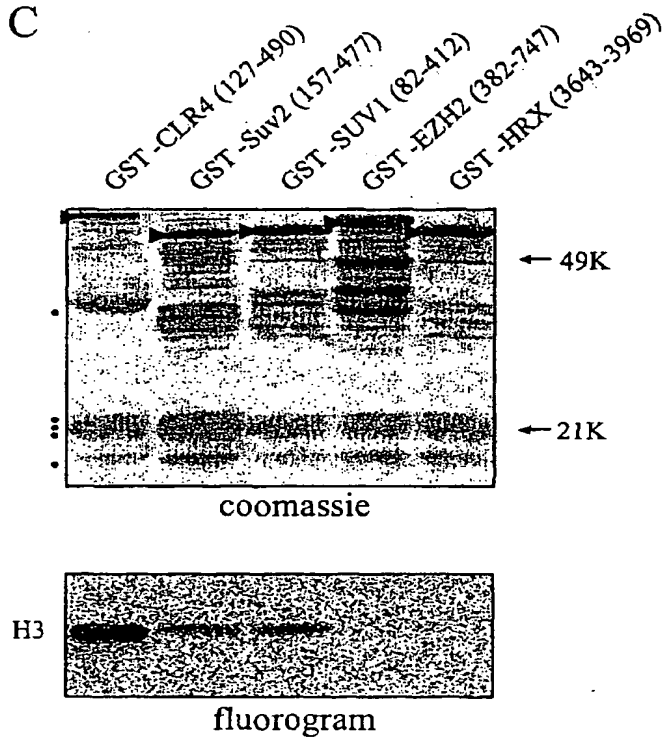


Fig. 3/1

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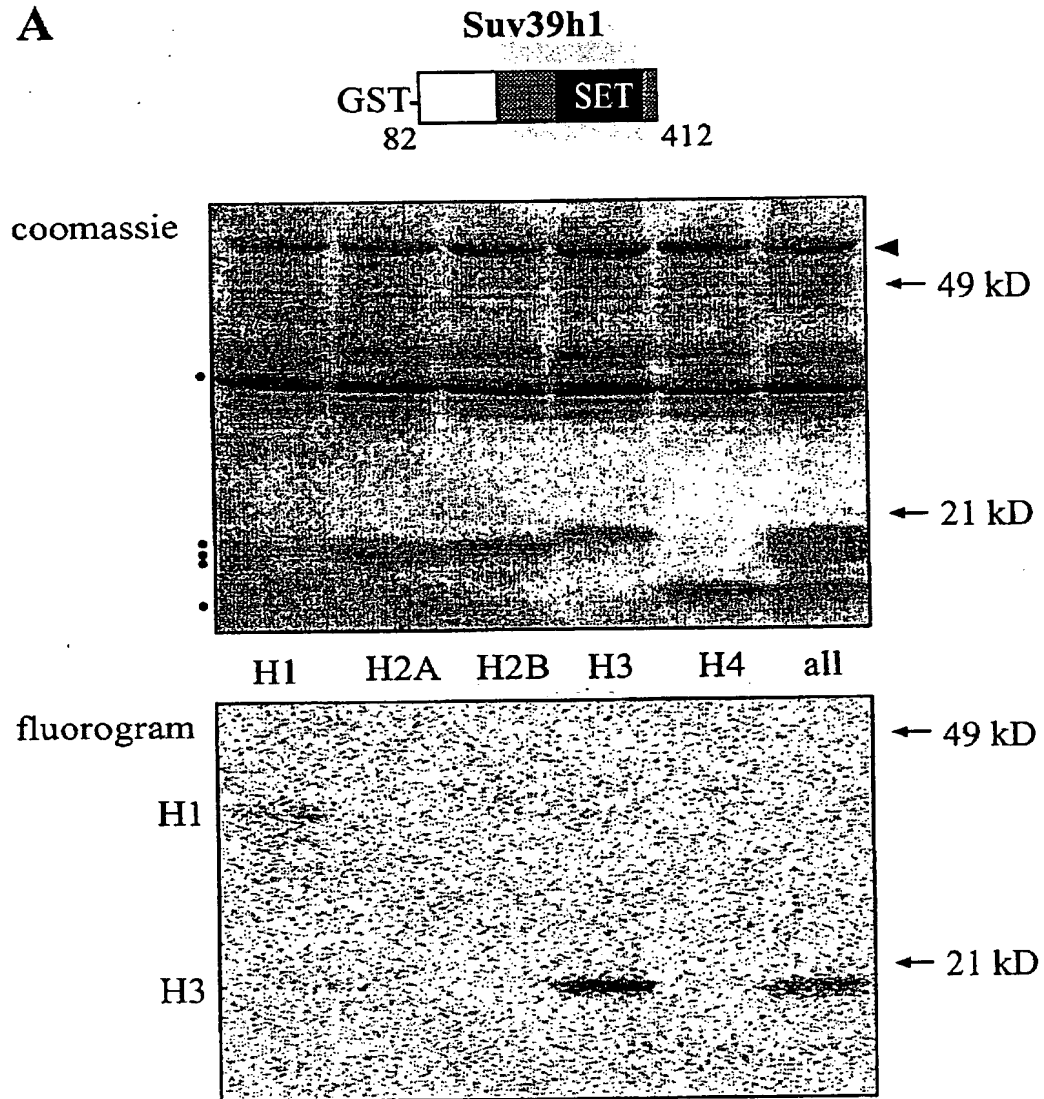
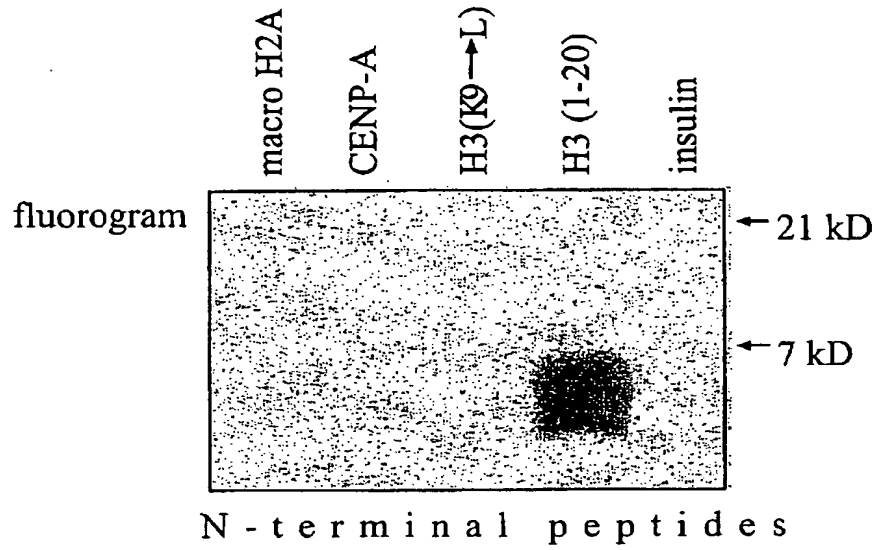


Fig. 3/2

B



C

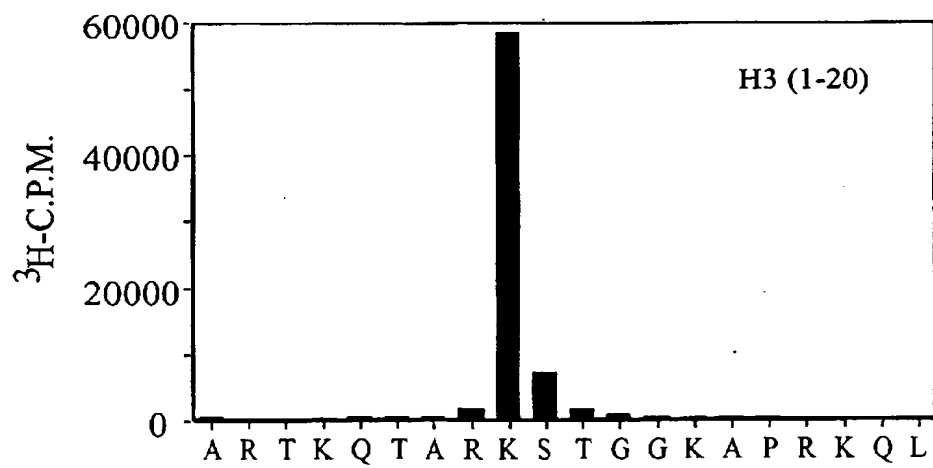


Fig. 4/1

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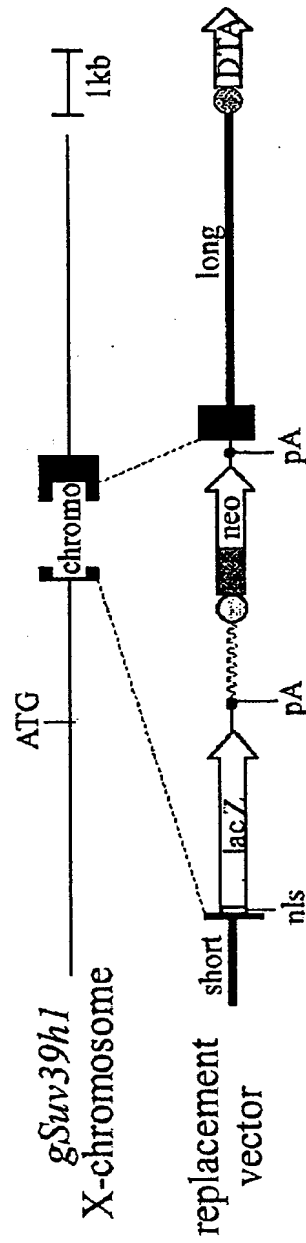


Fig. 4/2

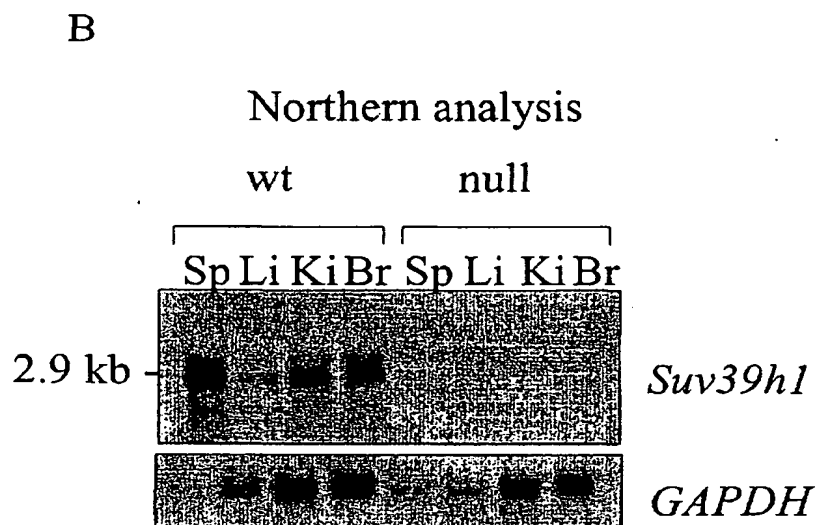
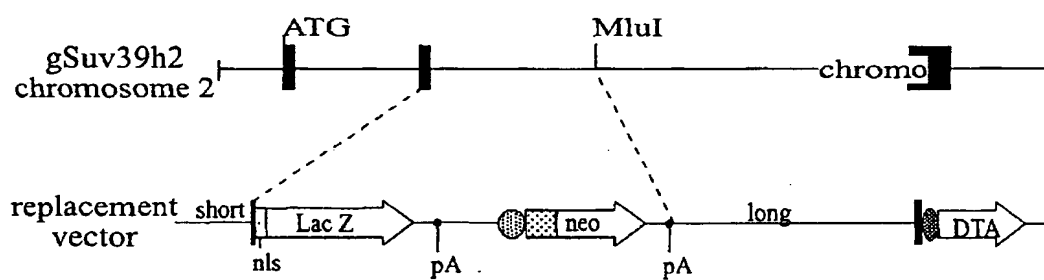


Fig. 4/3

C



Western analysis
(testis extracts)

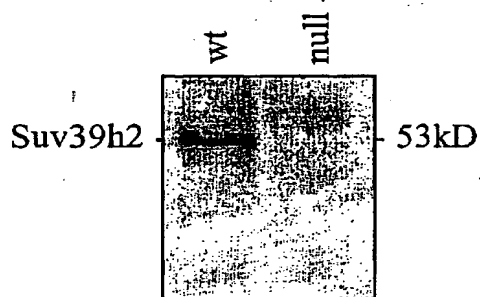


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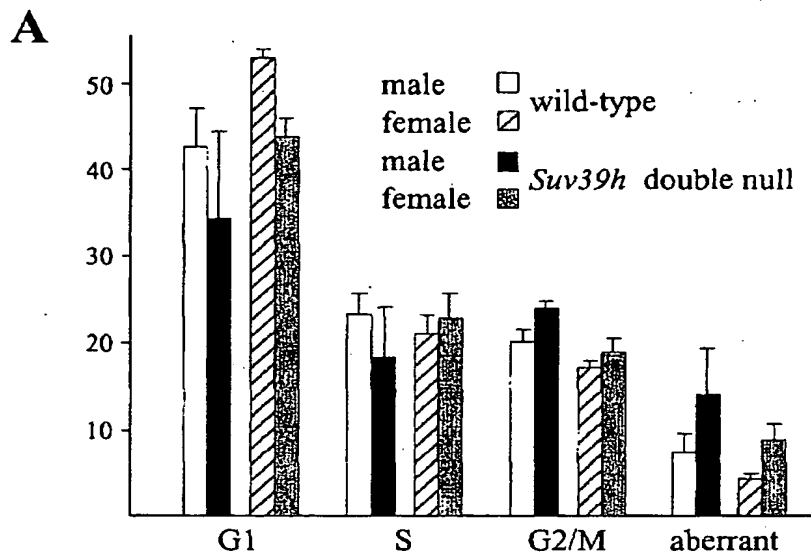


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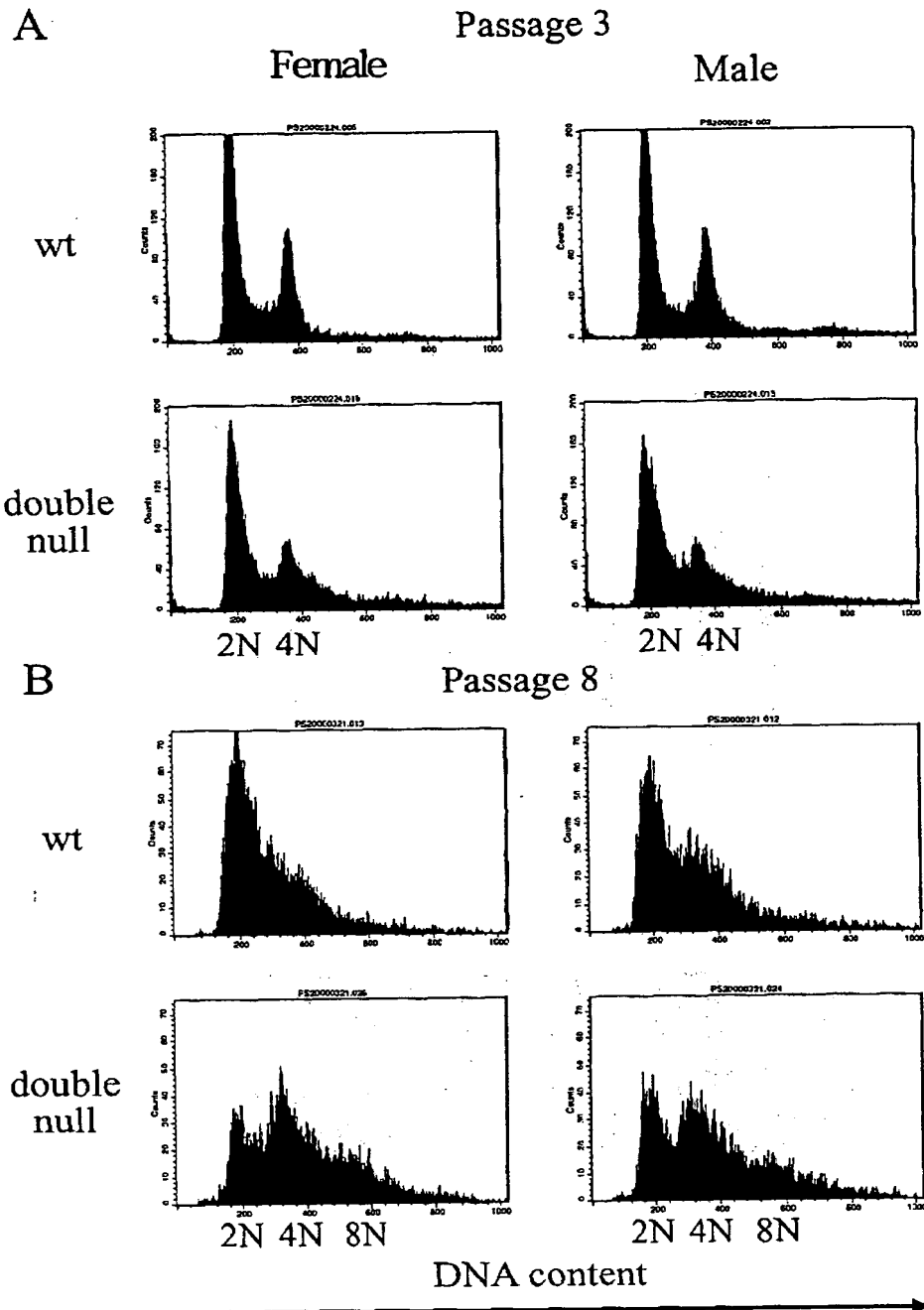
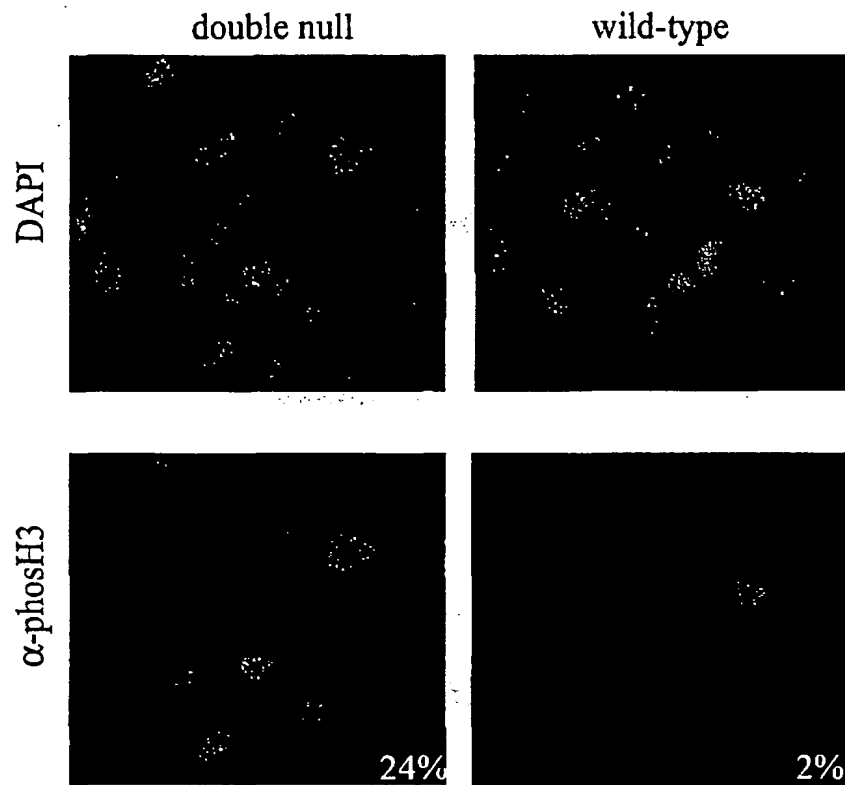


Fig. 7

A



B

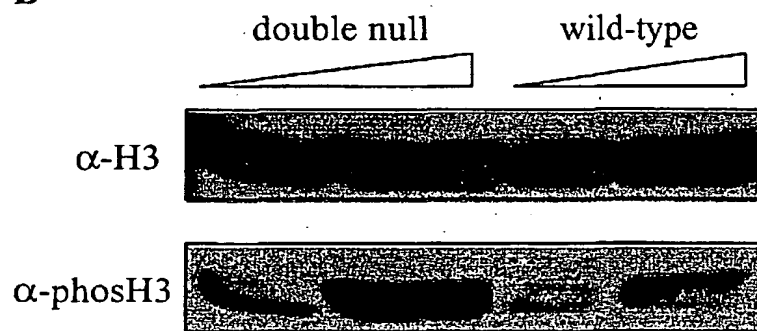
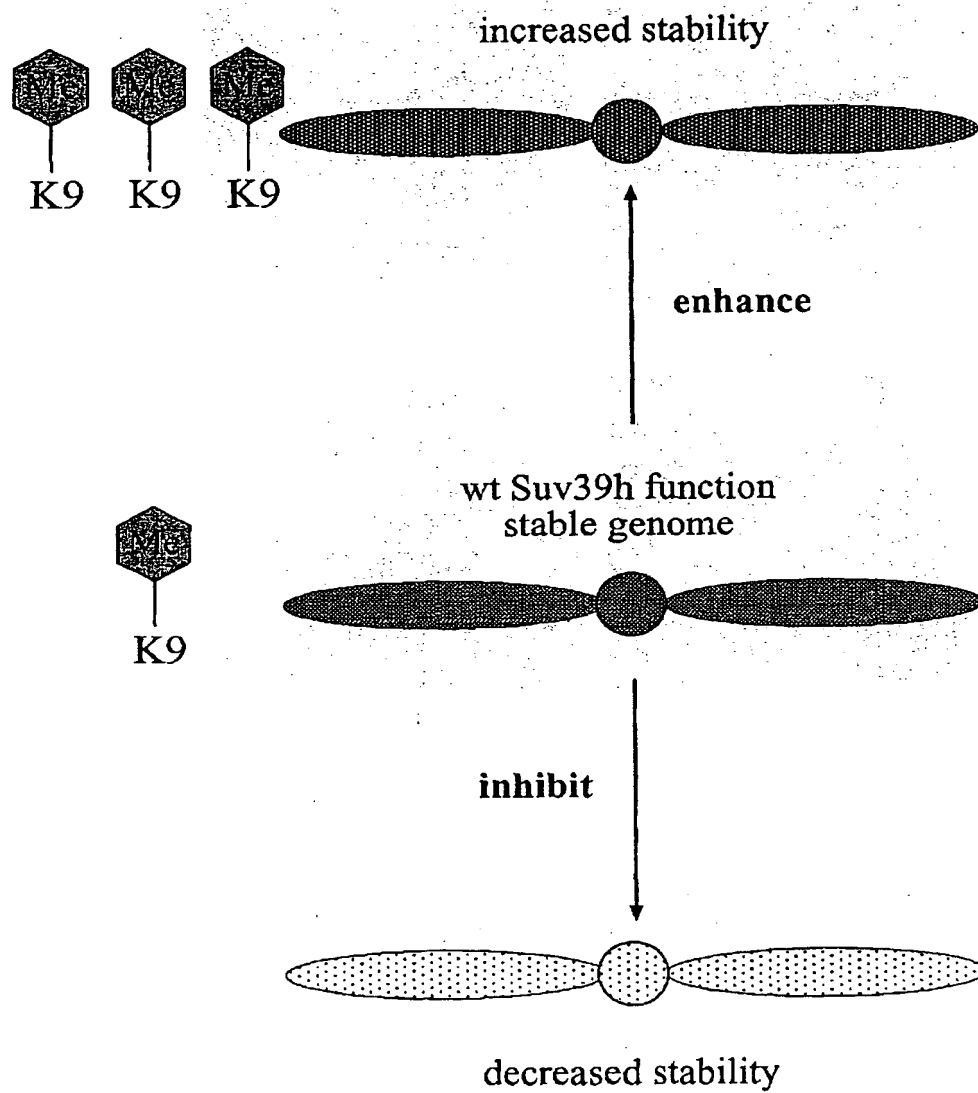


Fig. 8



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European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

EP 00 11 2345

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.CI.7)
X	WALLWORK J C ET AL: "PROPERTIES OF SOLUBLE RAT BRAIN HISTONE LYSINE METHYL TRANSFERASE EC-2.1.1.25" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 252, no. 17, 1977, pages 5977-5980, XP000974350 EN ISSN: 0021-9258 * abstract *	1,6,8	C12Q1/48 G01N33/68
X	VENKATESAN M ET AL: "PARTIAL PURIFICATION AND CHARACTERIZATION OF A PROTEIN LYSINE METHYL TRANSFERASE EC-2.1.1.43 FROM PLASMODIA OF PHYSARUM-POLYCEPHALUM" BIOCHEMISTRY, vol. 18, no. 24, 1979, pages 5365-5371, XP002154903 ISSN: 0006-2960 * abstract *	1,6,8	
			TECHNICAL FIELDS SEARCHED (Int.CI.7)
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INCOMPLETE SEARCH			
<p>The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.</p> <p>Claims searched completely:</p> <p>Claims searched incompletely:</p> <p>Claims not searched:</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p>			
Place of search		Date of completion of the search:	Examiner
THE HAGUE		22 December 2000	Tuynman, A
CATEGORY OF CITED DOCUMENTS		<p>T: theory or principle underlying the invention</p> <p>E: earlier patent document, but published on, or after the filing date</p> <p>D: document cited in the application</p> <p>L: document cited for other reasons</p> <p>.....</p> <p>&: member of the same patent family, corresponding document</p>	
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INCOMPLETE SEARCH
SHEET C

Application Number
EP 00 11 2345

Claim(s) searched completely:
1-10

Claim(s) not searched:
11

Reason for the limitation of the search:

Present claim 11 relates to compounds defined by reference to a desirable characteristic or property, namely being identifiable by the methods of claims 1 to 9.

The claim covers all compounds having these characteristics or properties, whereas the application provides no support within the meaning of Article 84 EPC nor disclosure within the meaning of Article 83 EPC. In the present case, the claim so lacks support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claim also lacks clarity (Article 84 EPC). An attempt is made to define the method and compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for this claim.



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PARTIAL EUROPEAN SEARCH REPORT

Application Number

EP 00 11 2345

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
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D,A	AAGAARD LOUISE ET AL: "Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 18, no. 7, 1 April 1999 (1999-04-01), pages 1923-1938, XP002154905 ISSN: 0261-4189 * abstract * * page 1925; figure 1 *	1,2,4,6,8	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
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EPO FORM 1503 03.02 (P04C10)



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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
T	REA STEPHEN ET AL: "Regulation of chromatin structure by site-specific histone H3 methyltransferases." NATURE (LONDON), vol. 406, no. 6796, August 2000 (2000-08), pages 593-599, XP002154907 ISSN: 0028-0836 * the whole document *	1,2,4-6, 9,10	
A	KLEIN ROBERT R ET AL: "Cloning and developmental expression of pea ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit N-methyltransferase." PLANT MOLECULAR BIOLOGY, vol. 27, no. 2, 1995, pages 249-261, XP002154908 ISSN: 0167-4412 * abstract *	1-3	
X	VON HOLT ET AL.: "Isolation and Characterization of Histones" METHODS IN ENZYMOLOGY, vol. 170, 1989, pages 431-523, XP000972597 * page 516 *	10	TECHNICAL FIELDS SEARCHED (InCL7)
A		1,4,5,9	
A	MACFARLEN J O ET AL: "COLORIMETRIC ASSAY FOR GUAIACOL O METHYL TRANSFERASE" JOURNAL OF PHARMACEUTICAL SCIENCES, vol. 65, no. 11, 1976, pages 1689-1690, XP002156114 ISSN: 0022-3549 * abstract *	1,7	
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EPO FORM 183 CL.7 (PAC19)

EP 1 162 274 A1



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DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	<p>WO 99 11667 A (RAYMACKERS JOSEPH ; UNION ANN (BE); INNOGENETICS NV (BE); MEHEUS LY) 11 March 1999 (1999-03-11) * abstract * * page 4, line 15 - line 22 * * page 5, line 27 - page 6, line 4 *</p>	1,9	
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EPO FORM 1803 01.92 (P04C10)

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**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 00 11 2345

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The members are as contained in the European Patent Office EDP file on
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22-12-2000

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		EP 0944649 A	29-09-1999

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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